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Prevalence and Genotypes of GB Virus C/Hepatitis G Virus (GBV-C/HGV) and Hepatitis C Virus Among Patients Infected With Human Immunodeficiency Virus: Evidence of GBV-C/HGV Sexual Transmission

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The development of new antiretroviral agents may improve survival of HIV-infected individuals, and therefore chronic viral hepatitis may become more relevant in these patients. The presence of GBV-C/HGV and hepatitis C virus (HCV) RNA were investigated by reverse transcriptase-nested polymerase chain reaction in plasma from 168 Spanish HIV-infected patients belonging to four different risk groups: intravenous drug users (IVDUs), hemophiliacs, homosexuals, and heterosexuals. GBV-C/HGV-RNA and HCV-RNA were detected in 18% and 43% of the patients, respectively. The prevalence of current infection with these viruses was notably high, 19% for GBV-C/HGV and 69% for HCV, among individuals with parenteral risk of infection (intravenous drug abusers and hemophiliacs), but sexual transmission with GBV-C/HGV was also suggested because 16.5% of patients with sexual risk, either homosexual or heterosexual, had GBV-C/HGV-RNA in plasma. Although investigation of GBV-C/HGV-RNA possibly underestimates the actual prevalence of infection with GBV-C/HGV, the above data suggest that sexual contact may play a relevant role in the spread of this virus. Phylogenetic analysis showed no evidence for clustering of NS3 sequences into different genotypes or subtypes of GBV-C/HGV. ***J. Med. Virol. 55:293–299, 1998.***

KEY WORDS: viral hepatitis; AIDS; phylogenetic analysis; route of transmission; sexual contact

INTRODUCTION

The GB virus C/hepatitis G virus (GBV-C/HGV) is a virus isolated recently and belongs to the *Flaviviridae* family [Simons et al., 1995; Leary et al., 1996; Linnen et al., 1996]. GBV-C/HGV can be detected in a relatively high proportion of apparently healthy volunteer blood donors [Simons et al., 1995; Linnen et al., 1996; Masuko et al., 1996; Saiz et al., 1997a], in persons exposed to parenterally transmitted viruses, such as recipients of blood transfusions [Schmidt et al., 1996; Tagariello et al., 1996; Alter et al., 1997a; Jarvis et al., 1997], patients on hemodialysis [Masuko et al., 1996; Forns et al., 1997], and intravenous drugs abusers [Aikawa et al., 1996; Schreirer et al., 1996; Stark et al., 1996; Fiordalisi et al., 1997], and also in patients with acute and chronic liver disease [Aikawa et al., 1996; Schreirer et al., 1996; Tagariello et al., 1996; Tanaka et al., 1996; Alter et al., 1997a, 1997b; Saiz et al., 1997a; Guilera et al., 1998], including those with fulminant hepatic failure [Heringlake et al., 1996; Yoshiba et al., 1996; Kanda et al., 1997; Saiz et al., 1997b]. Although parenteral transmission of GBV-C/HGV is well documented, other routes of infection such as vertical [Feucht et al., 1996; Lin et al., 1996] and sexual [Stark et al., 1996; Fiordalisi et al., 1997; Kao et al., 1997] have also been suggested, but the efficiency of these mechanisms in the transmission of this virus is still unknown.

Despite the quasispecies nature of GBV-C/HGV, the genetic heterogeneity of this virus is lower than that of hepatitis C virus (HCV) [Muerhoff et al., 1996; Pickering et al., 1997]. Analysis of the E2, NS3, or NS5b region sequences did not identify the existence of GBV-C/HGV genetics groups or types [Muerhoff et al., 1997]. However, based on sequence heterogeneity of the 5′ noncoding region (5′NCR), or longer coding regions, at

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least three different types of GBV-C/HGV variants have been described [Fukushi et al., 1996; Muerhoff et al., 1996, 1997; Khudyakov et al., 1997; Okamoto et al., 1997; Smith et al., 1997]. Although infection with certain strains of GBV-C/HGV in patients with fulminant hepatic failure [Heringlake et al., 1996] has not been confirmed, the possibility that strain variation might be related to the pathogenicity of GBV-C/HGV cannot be disregarded.

After implementation of new combined therapies for the treatment of HIV infection [Ho et al., 1995], other associated long-lasting diseases (such as chronic viral hepatitis) are gaining relevance in the prognosis of these patients. Since GBV-C/HGV, HCV, and HIV are transmitted through similar routes, we analyzed the prevalence and route of transmission of GBV-C/HGV and HCV in plasma from 168 Spanish HIV-infected patients belonging to four different risk groups: intravenous drug users (IVDUs), hemophiliacs, homosexuals, and heterosexuals. The presence of HCV and GBV-C/HGV in plasma was detected by specific reverse transcriptase (RT)-nested polymerase chain reaction (PCR). HCV genotype and nucleotide sequence heterogeneity of GBV-C/HGV were investigated and a phylogenetic analysis of the GBV-C/HGV-NS3 gene was also carried out.

PATIENTS AND METHODS

Patients

One hundred and sixty-eight HIV-infected patients were studied. This figure includes 41 intravenous drug users (14 women and 27 men), 43 hemophiliacs (1 woman and 42 men), 41 homosexual men, and 43 heterosexuals (35 women and 8 men). Age ranged from 14 to 61 years (mean 35 years). At the time of plasma collection, the median $CD4^+$ count was 312 ± 230 (range 10–1,050) and 95 patients (56%) had elevated serum alanine aminotransferase (ALT). Plasma samples were collected from January 1993 to May 1997 and immediately stored at −70°C. Patients were interviewed in detail about risk behavior by using standardized questionnaires and their informed consent was obtained. The study was approved by the Ethics Committee of our Institutions.

Blood Donors and Control Patients

Presence of GBV-C/HGV-RNA was also investigated in 200 consecutive, first donation, volunteer blood donors [Saiz et al., 1997a]. One hundred and fourteen were men and 86 women. Their mean age was 39 years, ranging from 18 to 65. None had markers of HIV infection (Cobas Core, Anti-HIV1/HIV2 EIA, DAGS, Roche Diagnostic System, Inc., Branchburg, NJ). HBsAg (HBsAg ELISA Test System 3, Ortho Diagnostics System, Raritan, NJ) was detected in one donor. The anti-HCV test (anti-HCV 3rd Generation ELISA Test, Ortho Diagnostics System) was positive in two donors, in whom the RIBA-3 test (Chiron Riba HCV 3.0, Ortho Diagnostic System, Raritan, NJ, USA) was negative in one case and indeterminate in the other.

Serum samples from patients with previously documented GBV-C/HGV infection [Saiz et al., 1997a] were used as control in amplification and sequencing experiments.

RNA Extraction

RNA was extracted from a volume of 100 μl of plasma from HIV-infected patients as described by Boom et al. [1990], based on guanidinium-thiocyanate lysis buffer and glass milk. Extracted RNA was resuspended in 400 μl of TE buffer. RNA was extracted from a volume of 140 μl of sera from volunteer blood donors and GBV-C/HGV-infected control patients as previously described [Saiz et al., 1997a].

HCV-RNA Analysis and Genotyping

For detection of HCV-RNA, reverse transcription and nested PCR were carried out as previously described [Saiz et al., 1997a] using specific oligonucleotides of the well-conserved 5′NC region, except that 50 μl of RNA was used in a final volume of 100 μl. Specific PCR products were identified by agarose gel electrophoresis. Appropriate negative and positive controls were included in each round of reactions to ensure specificity and, to prevent contamination, Kwok and Higuchi [1989] guidelines were strictly observed in both HCV-RNA and GBV-C/HGV-RNA analyses. Genotyping was performed by restriction fragment length polymorphism (RFLP) of the amplified 5′NCR according to the method described by Davidson et al. [1995].

GBV-C/HGV-RNA Analysis

GBV-C/HGV-RNA was detected by RT and nested PCR. Reverse transcription and cDNA synthesis were carried out in a single-step reaction using 50 μl of extracted RNA and the specific oligonucleotides GNS3-1 and GNS3-2 (derived from the NS3 gene of GBV-C/HGV) at a final concentration of 0.25 μM, in a volume of 100 μl of PCR buffer (10-mM Tris-HCl, pH 8.3, 50-mM KCl, 1.5-mM $MgCl_2$, 0.01% gelatin, 200 μM each deoxynucleotide) with 2.5 U of AMV-RT (Promega, Madison, WI), 10 U of ribonuclease inhibitor (RNAsin, Promega), and 1.25 U of Taq DNA polymerase (Gibco-BRL, Gaithersburg, MD). The reaction was performed at 42°C for 30 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, and a final extension step of 8 min at 72°C, in a Perkin-Elmer-Cetus DNA thermal cycler. A nested PCR was carried out using 5 μl from the RT-PCR first amplification reaction in a final volume of 50 μl of PCR buffer with 1.25 U of Taq DNA polymerase and oligonucleotides GNS3-3 and GNS3-4 using 30 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, with a final step of 8 min at 72°C. Control for specificity and prevention of contamination were performed as described above for HCV-RNA amplification.

TABLE I. Prevalence of Coinfections Wirh GBV-C/HGV and HCV Among HIV-Infected Patients According to Risk Factors of Exposure

Risk factor (n)	GBV-C/HGV	HCV	GBV-C/HGV and HCV	GBV-C/HGV alone	HCV alone	None
Parenteral exposure (84)	16 (19%)	58 (69%)	12 (14%)	4 (45%)	46 (55%)	22 (26%)
IVDU (41)	11 (27%)	28 (68%)	10 (24%)	1 (2.5%)	18 (44%)	12 (29%)
Hemophilia (43)	5 (11.5%)	30 (70%)	2 (4.5%)	3 (7%)	28 (65%)	10 (23%)
Sexual exposure (84)	14 (16.5%)	14 (16.5%)	1 (1.2%)	13 (15%)	13 (15%)	57 (68%)
Homosexual (41)	8 (19%)	6 (14%)	1 (2.5%)	7 (17%)	5 (12%)	28 (68%)
Heterosexual (43)	6 (14%)	8 (18.5%)	0	6 (14%)	8 (18.5%)	29 (67%)
Total (168)	30 (18%)	72 (43%)	13 (7.5%)	17 (10%)	59 (35%)	79 (47%)

GBV-C/HGV-NS3 primers (GNS3-1, GNS3-2, GNS3-3, GNS3-4) were specifically designed based on previously reported full-length sequences of GBV-C/HGV isolates [Leary et al., 1996; Linnen et al., 1996]. The sequences of the primers are as follows: GNS3-1, 5′-GACGTTGGTGAGATTCCCTT (nucleotide positions 4257 to 4276); GNS3-2, 5′-GTAATGGTGGGATCAAGGGT (nucleotide positions 4542 to 4561); GNS3-3, 5′-GGTGGAATTCCCTTTTATGG (nucleotide positions 4263 to 4282, with an *Eco*RI site underlined); and GNS3-4, 5′-GGTGGGATCCAGGGTCACCT (nucleotide positions 4537 to 4556, with a *Bam*HI site underlined). Nucleotide positions are according to that of GBV-C/HGV strain reported by Linnen et al. [1996].

Primers were tested for specificity on randomly selected negative and positive samples from previous studies where 5′NCR primers were used [Forns et al., 1997; Saiz et al., 1997a, 1997b; Guilera et al., 1998]. In all cases the concordance with previous results was complete.

GBV-C/HGV Sequencing

DNA-amplified products encoding the GBV-C/HGV-NS3 region were purified using the QIAquick PCR purification kit (Quiagen GbmH, Hilden, Germany) and sequenced with primer GNS3-3 using an Applied Biosystem 310 DNA Sequencer. Sequence editing was performed using Sequence Navigator (Applied Biosystems, Foster City, CA).

Sequence Alignment and Phylogenetic Analysis

Sequences were aligned using CLUSTAL W [Thompson et al., 1994]. Phylogenetic reconstructions were generated by using neighbor-joining in the Phylogeny Inference Package (PHYLIP) [Felsenstein, 1988, 1995], with Kimura two-parameter distance matrix and a ratio of transition to transversion of 2.0 (programs DNADIST and NEIGHBOR). Bootstrap resampling [Felsenstein, 1985] was applied to the neighbor-joining trees (programs SEQBOOT and CONSENSE) to assign approximate confidence limits to individual branches. In order to increase confidence in the reconstructed phylogenies, phylogenetic analysis was also conducted with Fitch-Margoliash, Maximun likelihood, and Parsimony methods (programs FITCH, DNAML, and DNAPARS, respectively) [Felsenstein, 1995]. The GBV-C/HGV sequences have been submitted to Genbank under accession numbers AF014847 to AF014879.

Statistical Analysis

Comparison between groups were made by the chi-square or Fisher's exact methods for categorical variables.

RESULTS

HCV-RNA was detected in 72 patients (43%) with HIV infection but in none of 200 volunteer blood donors ($P < 0.0001$). GBV-C/HGV-RNA was detected in 30 patients (18%) with HIV infection and in 6 blood donors (3%) ($P < 0.0004$). Thus, current infection with HCV was more frequent ($P = 0.0004$) than current infection with GBV-C/HGV in HIV-infected patients. The prevalence of GBV-C/HGV infection was almost identical in HCV-RNA-positive (13/72, 18%) and in HCV-RNA-negative patients (17/96, 17.7%).

The prevalence of infection with GBV-C/HGV and HCV in HIV-infected patients in relation to the presumed source of infection is summarized in Table I. Among patients with parenteral risk of exposure, infection with HCV (69%) was more prevalent than infection with GBV-C/HGV (19%) ($P < 0.0001$). Fourteen percent of these subjects were coinfected with HCV and GBV-C/HGV and only 26% had no serologic evidence of current infection with these viruses. Among patients at sexual risk of exposure, the prevalence of current infection with HCV or GBV-C/HGV was identical (16.5%). Fifty-seven patients (68%) were not currently infected with HCV or GBV-C/HGV, and only one patient (1.4%) was coinfected with both viruses.

The prevalence of infection with HCV was significantly greater ($P < 0.00001$) in patients with parenteral (69%) than in those with sexual (16.5%) risk of exposure. In contrast, the proportion of patients with detectable GBV-C/HGV-RNA was similar in these two groups of patients. Infection with HCV alone was more frequent in patients with parenteral risk (55%) than in those with sexual risk of exposure (15%) ($P < 0.00001$). Infection with GBV-C/HGV alone was about three times more frequent in patients with sexual risk (15%) than in those with parenteral risk of exposure (4.5%) ($P = 0.01$). Among HCV-infected patients, infection with GBV-C/HGV was more frequent in patients with par-

TABLE II. Relationship Between Risk Factors of Exposure, Distribution of HCV Genotypes, and Presence of Infection With GBV-C/HGV in HIV Patients

Risk factor (n)	HCV genotype	HCV-infected	GBV-C/HGV-infected
Parenteral exposure (58)	1a	14 (24%)	1 (8%)
	1b	36 (62%)	7 (58%)
	non-1	8 (14%)	4 (34%)
IDVU (28)	1a	6 (21.5%)	1 (10%)
	1b	18 (64.5%)	5 (50%)
	non-1	4 (14%)	4 (40%)
Hemophilia (30)	1a	8 (26.5%)	0
	1b	18 (69%)	2 (100%)
	non-1	4 (13.5%)	0
Sexual exposure (14)	1a	3 (21%)	0
	1b	7 (50%)	1 (100%)
	non-1	4 (29%)	0
Homosexual (6)	1a	2 (33%)	0
	1b	4 (67%)	1 (100%)
	non-1	0	0
Heterosexual (8)	1a	1 (12.5%)	0
	1b	3 (37.5%)	0
	non-1	4 (50%)	0
Total (72)	1a	17 (24%)	1 (8%)
	1b	43 (60%)	8 (61%)
	non-1	12 (16%)	4 (31%)

enteral (12/58, 21%) than in those with sexual (1/14, 7%) risk of exposure.

Differences in the prevalence of current infection with either GBV-C/HGV or HCV in relation to the particular type of risk factor involved (use of intravenous drugs or blood products transfusion among patients at parenteral risk of exposure; homosexual or heterosexual exposure among those at sexual risk) were not observed. However, coexistent infection with HCV and GBV-C/HGV was most frequent in patients with history of IVDU (24%) ($P < 0.01$).

Investigation of HCV genotype in HCV-RNA-positive plasma disclosed infection with genotype 1a in 17 patients (24%), with genotype 1b in 43 (60%), and with genotypes other than 1 in 12 (16%), including genotype 2a/c and 2b in one case each, genotype 3a in four, genotype 4 in five, and genotype 5 in one. The relationship between HCV genotype, the presumed source of infection, and the presence of simultaneous infection with GBV-C/HGV is shown in Table II. Although accurate analysis was not possible due to the low number of cases, infection with HCV genotype 1b appeared to be more frequent than infection with other genotypes, irrespective of the source of exposure, except in patients with heterosexual risk of exposure. GBV-C/HGV infection did not appear to be associated with any particular HCV genotype.

At the time of sampling, abnormal serum ALT activity was recorded in 48 of the 59 (81%) patients infected with HCV alone (mean 92 UI/L, range 45 to 230) and in only three of the 17 (18%) infected with GBV-C/HGV alone (mean 53 UI/L, range 49 to 60).

A $CD4^+$ lymphocyte count below 200 cells per ml was recorded in 51% of patients with HCV coinfection and in only 6% of those coinfected with GBV-C/HGV alone.

Genetic heterogeneity of GBV-C/HGV was investigated by direct sequence analysis of GBV-C/HGV-NS3 PCR-amplified products obtained from 33 subjects infected with GBV-C/HGV, including 27 HIV-infected patients, 3 volunteer blood donors, and 3 HIV-negative control patients with chronic hepatitis C. When pairwise comparisons were done, most of the observed nucleotide distances were within the range of 85–93% (mean 89%) (data not shown). With the exception of five samples, most sequences differed from all others at a similar level of nucleotide distance. These observations indicate a similar distribution of genetic distances, suggesting that isolates from all the samples analyzed belong to the same type or subtype of GBV-C/HGV.

An unrooted phylogenetic tree for the 33 GBV-C/HGV-NS3 sequences was reconstructed by the neighbor-joining method in order to establish the evolutionary relationships among GBV-C/HGV isolates (data not shown). The evolutionary pattern of the NS3 sequences resembled a "star" phylogeny with branches radiating from the same point. Consistent topological patterns or clusters were not observed. There was substantial intermingling of viral sequences from different risk groups and from different isolation dates, with low bootstrap values representing different clusters. Only two groups of sequences showed high bootstrap values. Although samples within these two groups belonged to patients with the same risk factor of exposure, no epidemiological links were found among them. As stated above, no distinctive clustering of viral sequences with sampling time was observed. These findings were confirmed when the phylogenetic analysis was carried out by the Maximum likelihood and the Parsimony methods (data not shown).

To further investigate the evolutionary relationship between GBV-C/HGV isolates, a tree was derived by running the 33 GBV-C/HGV-NS3 sequences obtained in this study and 22 NS3 sequences reported previously and collected from 9 different countries (data not shown) [Leary et al., 1996; Linnen et al., 1996; Pickering et al., 1997]. The resulting tree showed a similar scenario to that of the tree described above, where no evidence for grouping of sequences into genetic types or subtypes was observed. Likewise, the branch lengths were similar among samples isolated in different continents and dates.

DISCUSSION

The recent availability of more effective antiretroviral agents for the treatment of HIV infection will presumably result in a substantial decrease of morbidity and mortality from AIDS-related complications in HIV-infected patients [Ho et al., 1995]. In this context, the impact of concurrent long-lasting diseases, such as chronic infection with hepatitis viruses, on the outcome of subjects infected with HIV is becoming increasingly relevant. Some studies noted that complications from

chronic liver disease are among the main causes of hospital admission in these patients [Stein et al., 1992]. In this study, the main features of HCV and GBV-C/HGV infections, two members of the *Flaviviridae* family that may cause chronic infection and liver disease in human, were examined in 168 Spanish patients infected with HIV.

In this cohort of patients, current infection with HCV (43%) was more prevalent than current infection with GBV-C/HGV (18%), confirming previous observations [Aikawa et al., 1996; Masuko et al., 1996; Schmidt et al., 1996; Stark et al., 1996; Tagariello et al., 1996; Alter et al., 1997a; Fiordalisi et al., 1997; Forns et al., 1997; Saiz et al., 1997a] that the prevalence of infection with these viruses was particularly high in patients with risk factors for parenteral transmission of bloodborne viruses, such as use of illicit intravenous drugs or transfusion with blood or blood products.

In this particular subgroup of patients, HCV-RNA was detected more frequently than GBV-C/HGV-RNA (69% vs. 19%). This difference may be explained by the remarkable tendency of HCV to cause persistent infection, whereas GBV-C/HGV infection frequently subsides after a relatively short period of time [Linnen et al., 1996; Schreirer et al., 1996; Stark et al., 1996; Alter et al., 1997b; Tacke et al., 1997]. Cross-protection against GBV-C/HGV infection provided by preexisting HCV infection might be an alternative explanation, as suggested recently [Stark et al., 1996]. However, this possibility is not supported by data from this study, where GBV-C/HGV-RNA was detected in a similar proportion of HCV-RNA-positive and -negative individuals (7.5% and 10%, respectively). Our data also contradict those from other studies [Aikawa et al., 1996] describing that HCV-positive individuals are at higher risk of being infected by GBV-C/HGV.

It has been suggested that HCV and GBV-C/HGV are transmitted together [Tagariello et al., 1996]. However, in the present study the prevalence of HCV infection in IVDUs and hemophiliacs was similar, whereas GBV-C/HGV-RNA was less frequently detected (27% vs. 11.5%) in the latter, in whom coinfection with both viruses was rare when compared with IVDUs (4.5% vs. 24%). Thus, our data indicate that each virus can be transmitted independently.

The distribution of HCV genotypes observed in the present study was quite similar to that described previously in non-HIV patients with chronic hepatitis C from our geographical area [López-Labrador et al., 1997; Saiz et al.,1997a, 1998]. However, as already reported [Saiz et al., 1997a], infection with HCV genotypes other than 1 appears to be more frequent in GBV-C/HGV-infected individuals.

Abnormal ALT serum levels were presented in only 18% of the patients infected with GBV-C/HGV, in marked contrast with the frequent ALT elevation observed in patients infected with HCV (81%), whether or not coinfected with GBV-C/HGV. Thus, GBV-C/HGV does not appear to be a major cause of liver injury in HIV-infected patients, in agreement with previous observations reported from our area [Forns et al., 1997; Saiz et al., 1997a, 1997b; Guilera et al., 1998] and elsewhere [Masuko et al., 1996; Stark et al., 1996; Tanaka et al., 1996; Alter et al, 1997a; Fiordalisi et al., 1997; Kanda et al., 1997].

The $CD4^+$ lymphocyte count was below 200 cell/ml in 51% of the patients with HCV, but in only 6% of those coinfected with GBV-C/HGV. This observation indirectly suggest that GBV-C/HGV-RNA is mainly detected in patients infected more recently with HIV, even though pertinent data were not available on the duration of HIV infection.

Epidemiological data indicate that GBV-C/HGV infection is mainly transmitted by the parenteral route, although a few studies have described that vertical transmission of GBV-C/HGV [Feucht et al., 1996; Lin et al., 1996] is also possible. Our results suggest that sexual transmission of GBV-C/HGV is also important in the spread of this virus. GBV-C/HGV-RNA was found in plasma from 14 patients (16.5%) with sexual risk for viral transmission, a proportion similar to that recently described by others [Stark et al., 1996; Fiordalisi et al., 1997]. No differences were observed between heterosexual and homosexual individuals concerning GBV-C/HGV infection.

It should be noted that many subjects included in the present study were immunocompromised, and this fact might have facilitated sexual transmission of GBV-C/HGV as previously suggested [Fiordalisi et al., 1997]. Notwithstanding, it should be remarked that GBV-C/HGV prevalence could be underestimated due to the development of humoral immunity [Tacke et al., 1997]; thus, it is possible that our data, based on the detection of GBV-C/HGV-RNA in plasma, do not accurately reflect the actual relevance of GBV-C/HGV transmission through sexual contact. In fact, interspousal transmission of this virus have been documented at the molecular level [Kao et al., 1997]. Thus, transmission of GBV-C/HGV by mechanisms other than parenteral exposure (such as sexual, vertical, or household contact) may explain the high proportion of GBV-C/HGV-RNA-positive individuals among volunteer blood donors observed in this and in other studies [Simons et al., 1995; Linnen et al., 1996; Masuko et al., 1996; Saiz et al., 1997a].

The phylogenetic reconstructions of the 33 GBV-C/HGV-NS3 sequences obtained in the present report from patients of the four risk groups did not show any specific genotype pattern by patient category (i.e., parenteral or sexual risk groups), ruling out the possibility of selective transmission of specific GBV-C/HGV genotypes. This was evidenced by the intermingling of viral sequences from the different groups, with low bootstrap values representing different clusters. Although there was genetic heterogeneity among the 33 isolates from Spain, pairwise comparisons between these sequences showed (except in five isolates) a comparable level of similarity. Interestingly, contemporary isolates displayed the same genetic distance observed in isolates from samples taken four years earlier, suggesting

that interpatient viral genetic diversification did not entail a net accumulation of nucleotide substitutions with time.

The alignment and the phylogenetic reconstruction using the 33 GBV-C/HGV-NS3 Spanish sequences and 22 sequences isolated worldwide did not differ from that obtained when only the Spanish isolates were used. Confidence intervals of the derived phylogeny were placed by application of the bootstrap resampling method, which takes into account uncertainties derived from the limited number of replacements between sequences [Martínez et al., 1992]. Thus, again, the low bootstrap values found in the tree did not support the existence of more than one GBV-C/HGV type. Since we have only analyzed a small segment of the genome, the existence of different GBV-C/HGV genotypes among these 33 Spanish isolates can not be ruled out. Recently, evidence for the existence of GBV-C/HGV genotypes based on analysis of the entire 5′NCR, or longer coding regions, has been reported [Fukushi et al., 1996; Muerhoff et al., 1996, 1997; Khudyakov et al., 1997; Okamoto et al., 1997; Smith et al. 1997]. In contrast, independent analysis of the E2, NS3, or NS5b region sequences did not identify the existence of GBV-C/HGV genetics groups or types [Muerhoff et al., 1997]. Although, major genotype differences are shown for many HCV subgenomic regions, this difference may be due to the relative conservation of GBV-C/HGV isolates in comparison to that between HCV types [Muerhoff et al., 1997; Pickering et al., 1997]. As opposed to HCV, GBV-C/HGV is not clearly implicated in the pathogenesis of liver disease [Alter, 1996; Masuko et al., 1996; Alter et al., 1997a, 1997b; Forns et al., 1997; Saiz et al., 1997a, 1997b; Guilera et al., 1998]. Then again, the interaction of each virus with the host is probably different and, therefore, the genetic evolution of both viruses may be subject to different selective constrains.

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